A MOLECULAR APPROACH TO PROMOTE WOUND HEALING IN THE SULFUR MUSTARD–EXPOSED HUMAN KERATINOCYTE MODEL

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ABSTRACT

Endogenous nitric oxide (NO) produced by three major NO synthases (NOSs) is a pleiotrophic signaling molecule in various tissue under normal and pathological conditions. In the skin, increased NO synthesized by inducible NOS (iNOS/NOS2), a NOS isozyme, has been considered to have pivotal roles in the inflammatory response to heat, infection and wound healing. It has also been shown that iNOS regulates the expression of several biomarkers, including Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES) and vascular endothelial growth factor (VEGF), to exert its effect in accelerating normal skin wound healing. However, there is conflicting evidence as to the roles of iNOS in the wound healing.

The effects of sulfur mustard (SM), a chemical warfare agent, on the skin include formation of blisters and slow-healing skin injuries due to delayed re-epithelialization. These are the major therapeutic targets of SM-caused skin damage. Re-epithelialization of the skin following SM injury is an important but challenging goal in treatment in order to protect the slow healing wound from infection.

The effects of SM on the expression of NOSs, VEGF and RANTES for wound healing were studied in an *in vitro* human skin model using normal human epidermal keratinocytes (NHEK). The cells were exposed to 1–20 μM SM immediately after scraping the monolayer of cells to wound them. SM delayed the healing of the wounded area, which, in an untreated (no SM exposure) cell layer, was closed (healed) through migration and proliferation of the cells by 48 h after scraping.

The expression of iNOS, RANTES, and VEGF was monitored by Western blotting and real-time RT-PCR. The levels of both protein and mRNA expression of iNOS and RANTES peaked at 7 h after wounding. Exposure to 20 μM SM abolished the enhanced expression at both the protein and mRNA levels. There were no significant changes in VEGF protein or mRNA expression levels. Fluorescence microscopy also showed strong iNOS expression 7 h after wounding in the NHEK, and SM substantially inhibited iNOS expression.

Taken together, these results suggest that iNOS has an important role in skin wound healing, and the prevention of SM-induced alterations in iNOS may be a potential treatment for SM skin injuries.

1. INTRODUCTION

Sulfur mustard (SM) is a chemical warfare agent that can cause skin burns, inflammation, and blisters (Saladi *et al*, 2006). Because there is no specific antidote thus far, treatment consists of addressing the symptoms—the injured skin exposed due to broken blisters must be closed by re-epithelialization as soon as possible to minimize the chances of infection and subsequent scarring of the denuded skin.

The process of wound repair in the skin is complex, involving dermal contraction and epithelial cell migration to repair the lesion and restore the skin's barrier properties. Prompting wound healing by re-epthelialization is the most challenging issue in the treatment of SM-injured skin, and identifying factors to improve wound healing after SM exposure is critical for new therapeutic strategies to improve the cure rate.

Re-epithelialization carried out by keratinocyte migration and proliferation is an important event in skin wound healing. Re-epithelialization is orchestrated by various growth factors produced by keratinocytes and other cutaneous cell types, including platelets, inflammatory cells, fibroblasts, and epithelial cells (Schwentker *et al*., 2002). Recent evidence clearly indicates that nitric oxide (NO) produced by NO synthase (NOS) followed by NO-mediated signaling in the skin

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plays a pivotal role in skin wound healing (Isenberg, 2005). However, the putative roles of NO in skin wound healing vary depending on the experimental models employed and/or the stages of wound healing studied. For example, in a rat model, suppression or deletion of inducible NOS (iNOS), the major producer of NO in the skin, showed delayed or lack of wound healing, respectively, and introduction of the iNOS gene to the wounded area rescued the delayed wound healing (Cals-Grierson, MM and Ormerod, AD, 2004). However, in contrast, NO production by iNOS was suppressed during wound healing in an *in vitro* model (Weller, R, 2003), suggesting a negative effect of NO on wound healing.

Among the many NO-regulated growth factors that participate in skin wound healing, vascular endothelial growth factor (VEGF) is a recently discovered endothelial growth factor (Yamasaki *et al*., 1998) and Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES) (Weller, 2003), a small chemotactic protein, have been shown to help skin wound healing. Most studies indicate that NO upregulates VEGF but downregulates RANTES expression (Kim *et al*, 2003). Keratinocytes at the wound edge express VEGF mRNA and have been shown to play a crucial role in cutaneous wound healing (Tonnesen, MG., *et al*., 2000). Formation of new blood vessels, which occurs through a combination of angiogenesis and vasculogenesis, plays a vital role in reparative processes such as wound healing. Molecules targeting VEGF or its receptor (VEGFR) could control wound healing (Harry *et al*., 2003). Although enhanced production of RANTES has been described in apoptotic dermatitis, in a mouse wound model this growth factor (a chemokine) produced by keratinocytes has a role in normal wound repair (Bryan, D., *et al*., 2005).

In this study, the effect of SM exposure on the expression of iNOS, VEGF and RANTES was studied to characterize the processes and molecular events that occur during wound healing using normal human epidermal keratinocytes (NHEK) *in vitro*.

2. EXPERIMENTAL PRCEDURES

2.1 Cell Culture and Wounding

NHEK were purchased from Cascade Biologics and cultured in Epilife medium containing the human keratinocyte growth supplement kit and antibiotics (Cascade Biologics) at 37°C and 10% CO_2. NHEK were propagated for two passages, and third-passage cells were used. For wound healing experiments, cells were seeded on collagen type I–precoated 90-mm culture dishes or coverslips, and grown to confluence. The medium was changed 24 h before wounding. The wound was made by scraping either a single (200-μl) pipette tip across the coverslips twice or an 8-channel pipette with 200-μl tips 15 times across the 90-mm dishes. Wound closure was followed by phase-contrast microscopy on a Nikon microscope, and digital images of the wound area were acquired as a function of time using a MicroFire™ (OLYMPUS) device and assessed by imaging software (Image-Pro Discovery).

2.2 Biomarker Expression

Expression of iNOS, RANTES and VEGF was analyzed by Western blotting using the respective mouse monoclonal antibodies (Abcom). The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce). Immunoreactivity was detected by chemiluminescence by addition of SuperSignal substrate solution (Pierce). The resulting bands were captured by a CCD camera system(FUJI: LAS-3000).

2.3 Real-time Reverse Transcription (RT)-PCR

For analysis of mRNA expression, total RNA was purified using the RNAqueous-4PCR® kit (Ambion) from cells at the end of the SM exposure time. Real-time PCR was performed in a two-step reaction using the High Capacity cDNA Archive kit (Applied Biosystems). The second step was performed in a fluorescence-monitoring temperature cycler (ABI-Prism 7500 Sequence Detection System, Applied Biosystems) with the TaqMan® Fast Universal PCR Master kit (Applied Biosystems) and specific primers for each of the genes purchased from Applied Biosystems. Every plate included a control gene (β-actin) for each experimental condition.

2.4 Immunofluorescence Staining

To visualize iNOS and nNOS (neuronal NOS/NOS1, constitutive), cells on coverslips were fixed in 4% paraformaldehyde, permeabilized in 0.2% (w/v) Triton X-100 in PBS, blocked with 0.5% horse serum in PBS, and incubated with the appropriate primary mouse monoclonal antibody (Abcam) at 4°C overnight. Cells were further incubated with rhodamine-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Labs). DAPI (Sigma) was used to counterstain nuclear DNA; the coverslips were then mounted with Antifade (Molecular Probes). Images were captured by a Bio-Rad 2000 laser confocal microscope.

2.5 Sulfur Mustard Exposure

For the SM exposure experiments, immediately after wounding, the regular medium of confluent NHEK was replaced with the same fresh medium containing concentrations of SM ranging from 1-20 μM and left in a chemical fume hood for 45 min at room temperature. Following the 45-min incubation in the fume hood, the

cultures were returned to the 37°C CO_2 incubator.

3. RESULTS

3.1 *In vitro* Wound Healing Model

First, to study the time frame for wound closure in this NHEK model, passage-three NHEK were grown to confluence on collagen type I–coated coverslips. When the cells were confluent, a wound was created by scraping the cell layer with a 200-µl pipette tip. The average width of the wound created by this method was 2.5 mm as measured by the imaging software. Wound healing was monitored under a phase microscope over time. As shown in Figure 1, about 12 h after wounding, cells started migrating toward the open space. The migrated cells started to proliferate at the site 24 h after scratching; the width of the wound at 24 h was reduced to 0.68 mm and the wound was completely closed within 48 h after wounding.

Figure 1. Establishment of an *in Vitro* Wound Healing Model Using Cultured NHEK.

Resurfacing by activated (wounded) NHEK at the wound edge. Cell migration and proliferation were monitored under a Nikon Phase Contrast microscope after scratching. Photomicrographs of NHEK are as shown: A, no scratch; B, 0 h; C, 12 h; D, 24 h; and E, 48 h after scratching. All images were captured by an Olympus MicroFire digital camera.

3.2 Effect of SM on Wound Healing

To study the effect of SM on wound healing, NHEK were exposed to various concentrations of SM ranging from 1 to 20 µM immediately after wounding. There was no delay in wound healing at concentrations of 1 and 5 µM SM compared to the no SM exposure samples (Fig. 2A and B vs. Fig. 1E); however, partial and complete inhibition was observed at 10 µM and 20 µM, respectively (Fig. 2C and D).

Figure 2. Dose-Response Effect of SM on Wound Healing.

NHEK were exposed to various concentrations of SM immediately after wounding, and healing was assessed 48 h later. Cells were exposed to 1 µM (A), 5 µM (B), 10 µM (C) or 20 µM (D) SM.

3.3 Expression of the three NOS Isoforms

It has been reported that all major NOS isoforms, that is, NOS1, NOS2 (iNOS), and NOS3, are present in the human skin, but only iNOS is inducible and strongly implicated in the production of high levels of NO in response to inflammation and external stimuli, such as heat. The level of NO produced by NOS1 or NOS3 is very low and considered to only have a role in the maintenance of normal skin function (in barrier function and in blood flow). Figure 3 shows the changes in expression levels of the three major NOS isoforms analyzed by western blotting during wound healing with or without exposure to 20 µM SM. In cells not exposed to SM, strong iNOS expression was observed 4 h after wounding compared to that of the control (no wounding), and this high level of iNOS expression was maintained for 24 h after scratching (Fig. 3). However, this strong expression, as measured by immunoreactivity with anti-iNOS, was suppressed by SM exposure. Expression of nNOS was also enhanced 4 h after wounding, but SM exposure did not suppress this enhanced level of nNOS. The levels of neuronal NOS (nNOS) were nearly undetectable regardless of SM exposure conditions (Fig. 3).

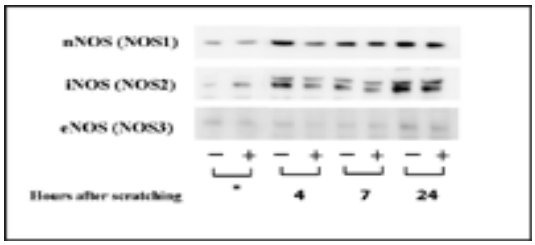

Figure 3. Time Course of Changes in Expression of the three NOS Isoforms after Scratching NHEK with or without SM Exposure.

NHEK were exposed to 20 µM SM immediately after wounding. At the times indicated, cells were collected for western blotting using antibodies against nNOS, iNOS and nNOS. *No scratching (control).

3.4 Flurescence Microscopy

The results obtained from this study thus far suggested that there were detectable levels of both nNOS and iNOS protein and mRNA in NHEK. iNOS and nNOS showed a marked increase in protein as well as mRNA levels, but the increased levels of nNOS mRNA were less than 50% of those of iNOS (Fig. 5). SM exposure resulted

in significant inhibition of especially iNOS protein and mRNA levels (Fig. 4 and 5). We observed an increase in iNOS and nNOS protein and mRNA levels, with a particularly marked increase for iNOS (Fig. 4 and 5). To confirm these results, confocal fluorescence microscopy was carried out to visualize iNOS and nNOS protein expression in NHEK with or without SM exposure. There was strong expression of iNOS that was substantially suppressed by SM exposure (Fig. 4, upper row). There were no significant changes in nNOS expression with or without SM exposure (Fig. 4, third row).

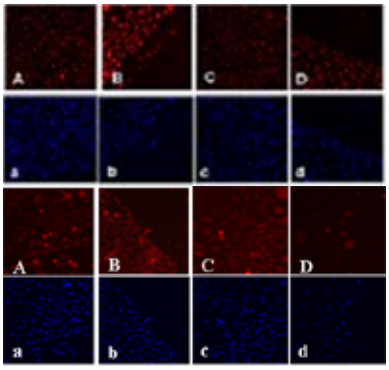

Figure 4. Immunofluorescence Staining of iNOS and nNOS in NHEK.

NHEK were fixed and permealized 7 h after scratching with or without 10 μM SM exposure. iNOS localization and expression levels were studied using monoclonal antibodies against iNOS and secondary rhodamine-conjugated mouse IgGs. DNA was stained with DAPI (a-d) simultaneously. The top row shows iNOS staining. The third row shows iNOS staining with SM exposure. The second row and bottom row show DAPI staining. A, Control (no scratching, no SM); B, 7 h after scratching with no SM exposure; C, 7 h of exposure to SM OR 7 h after SM exposure with no scratching; D, 7 h after scratching and SM exposure.

3.5 Expression of the three NOS Isoform mRNAs

To verify the protein expression levels shown in Figure 3, changes in mRNA levels of each NOS isozyme were studied using real-time PCR.

NHEK were exposed to 20 μM SM immediately after wounding. At the times indicated, cell samples were collected and total RNA was prepared followed by cDNA synthesis for use in real-time PCR. RQ: Relative Quantification. Expression was quantified relative to endogenous β-actin mRNA.

iNOS and eNOS mRNA expression increased 7 h and 24h, respectively, after wounding and the iNOS mRNA levels returned to the basal levels by 24 h. SM exposure (20 μM) suppressed this iNOS enhancement. There was no change in nNOS mRNA levels regardless of SM exposure.

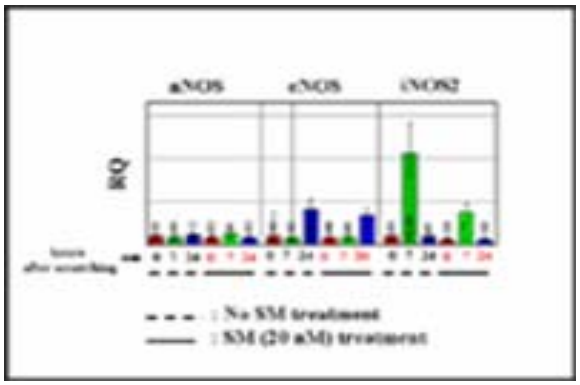

Figure 5. Time Course of Changes in Expression of the three NOS isozyme mRNAs.

3.6 Expression of RANTES and VEGF

Some reports have suggested that iNOS downregulates RANTES and upregulates VEGF expression. RANTES and VEGF protein levels were analyzed by western blotting up to 72 h after wounding with or without 20 μM SM exposure. As shown in Figure 5, the RANTES protein level increased 24 h after scratching and then sharply dropped to basal levels (compare with the no SM control); however, no increase in the expression of RANTES was observed with SM exposure. VEGF protein levels did not change significantly during the time course of the experiment regardless of SM exposure.

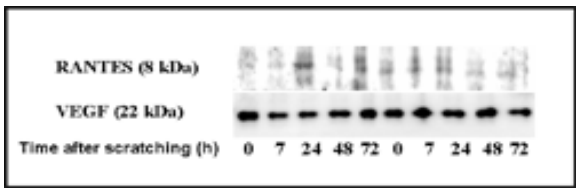

Figure 6. Protein Expression of RANTES and VEGF Following Wounding of NHEK.

RANTES protein expression peaked 7 h after scratching. SM exposure caused a marked decrease in RANTES mRNA expression compared with the control. There was no increase of RANTES expression among the SM treated samples after scratching. The RANTES protein expression peaked 7 h after scratching.

3.7 Expression of RANTES and VEGF mRNAs

As mentioned in Section 3.6, iNOS may downregulate RANTES and upregulate VEGF expression. To assess whether that is true in this NHEK model, mRNA expression of both RANTES and VEGF was monitored during wound healing up to 72 h after scratching (Fig. 7). RANTES mRNA was enhanced 7 h after scratching and returned to the basal level by 24 h, and SM exposure inhibited the enhancement. This suggests that RANTES mRNA expression either was not regulated or was upregulated by iNOS, contradictory to some of the published data. The VEGF mRNA decreased 24 h after scratching both with and without SM exposure (Fig. 6).

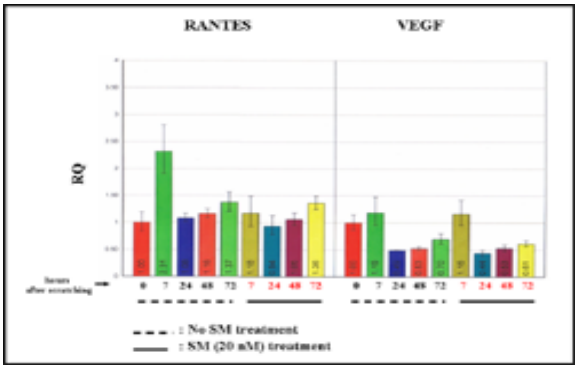

Figure 7. Time Course of Changes in Expression of RANTES and VEGF mRNAs.

NHEK were exposed to 20 μM SM immediately after wounding. At the times indicated, cell samples with or without SM exposure were collected and total RNA was prepared for use in real-time PCR.
RQ: Relative Quantification. Expression was quantified relative to endogenous β-actin mRNA.

4. DISCUSSION

The discovery of the multiple pathophysiological processes in which NO is involved has promising implications for the development of new drugs that regulate production of NO directly (NO-releasing) and/or indirectly (NO-inhibiting) for therapeutics. Most published findings support NO's important roles in accelerating skin wound healing. However, conflicting results that NO causes damage and delays skin wound healing have also been reported. All three major NOSs, nNOS (NOS1), iNOS (NOS2), and eNOS (NOS3) are found in the skin. The amount of NO produced by the constitutive nNOS and eNOS in the tissues is relatively small, and the inducible iNOS is responsible for the majority of NO production, indicating that iNOS induction plays an important role in the NO response in tissue.

It is not yet fully understood whether the SM-induced delay of wound healing in the skin as a result of reduced NO production at the wound site is due to inhibition of iNOS induction and/or to reduced activity of iNOS. Thus, a NHEK *in vitro* wound healing model was developed to begin to address this issue. We observed that exposure to 10-20 μM SM resulted in an apparent delay in wound healing in this model. A marked induction of iNOS was observed during normal wound healing, but exposure of NHEK to SM resulted in a substantial decrease in iNOS induction. These data strongly suggest that iNOS induction that is presumably followed by the release of a large amount of NO at the wound site plays an important role in wound healing in the *in vitro* NHEK wound model.

Based on our results, iNOS seems to upregulate the expression of the RANTES gene but not that of VEGF, which is inconsistent with previously published findings. Further study is necessary to confirm this hypothesis by manipulating the iNOS gene through knockdown and/or overexpression.

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CONCLUSIONS

(1) When a wound was created on a confluent NHEK culture by scratching with pipette tips, wound healing, as measured by closure of the opening via cell migration and proliferation, was complete 48 h after scratching.

(2) Exposure of cells to 20 μM SM immediately after wounding resulted in complete inhibition of wound healing.

(3) iNOS expression was enhanced and peaked 7 h after wounding, and SM inhibited this enhanced expression.

(4) Preventing SM-induced inhibition of iNOS may be a prospective strategy to promote wound healing in SM-exposed skin.

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